Chronic exogenous hyperinsulinaemia-induced hypertension in pregnant rats: effect of chronic treatment with L-arginine

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ABSTRACT

Recent studies have shown that maternal hyperinsulinaemia is a risk factor for the development of hypertension in pregnancy. Experimentally, pregnant rats with chronic exogenously induced hyperinsulinaemia (P-INS rats) have increased blood pressure at the end of gestation. This is associated with a blunted elevation of the urinary metabolites of nitrate (UNO\textsubscript{x}). In the present study, we aimed to evaluate the mechanism(s) of the increase in blood pressure in this model. Four groups were studied: normal pregnant rats (P rats), P-INS rats, P-INS rats treated with L-arginine (2 g/l in the drinking water) (L-ARG rats) and hyperinsulinaemic virgin rats (V-INS rats). Systolic blood pressure (SBP), UNO\textsubscript{x} excretion (on ingestion of a controlled low-nitrate diet), urine noradrenaline (norepinephrine) and plasma endothelin levels were evaluated. Rats were killed on day 22 of pregnancy. Five P-INS rats were not killed at this time, in order to measure SBP 30 and 60 days after delivery. Fetal number and fetal body weight were evaluated. At the end of pregnancy, a 10±3% increase in SBP was found in P-INS rats, contrasting with a fall of -15±4% in P rats (P < 0.01). In the L-ARG group at the end of pregnancy, SBP values had fallen by -14±2%, to values comparable with those of P rats. The increase in UNO\textsubscript{x} excretion was 175±38% in P rats, 106±12% in L-ARG rats and 41±8% in P-INS rats (P < 0.01 compared with P and L-ARG groups). No differences were found in the urinary excretion of noradrenaline or in the plasma levels of endothelin-1 between the pregnant groups. Fetal number was similar in all groups, but fetal body weight was lower for P-INS rats compared with P and L-ARG rats. Thus the blood pressure response to L-arginine strongly suggests that a decrease in NO availability may be the main pathogenic mechanism involved in the development of hypertension in this model.

INTRODUCTION

Recent studies have shown that maternal hyperinsulinaemia is a risk factor for the development of hypertension in pregnancy [1]. In an experimental model of chronic exogenously induced hyperinsulinaemia, we have shown that hyperinsulinaemic pregnant rats show increased blood pressure at the end of gestation, which is associated with a blunted elevation of the urinary excretion of nitrate [2]. In the present study, we aim to...

Key words: hyperinsulinaemia, hypertension, nitric oxide, pregnancy.

Abbreviations: P rats, normal pregnant rats; V-INS rats, virgin rats treated chronically with insulin; P-INS rats, pregnant rats treated chronically with insulin; l-ARG rats, P-INS rats treated with l-arginine from day 11 of gestation; SBP, systolic blood pressure; UNO\textsubscript{x}, urinary metabolites of nitrate; ET-1, endothelin-1; df, degrees of freedom.

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evaluate the mechanism(s) behind the increased blood pressure in this model.

MATERIALS AND METHODS

Female Wistar rats (body weight 200–280 g) were fed on a normal diet (0.35 g of NaCl, 20 g of protein and 1.17 g of arginine per 100 g) and allowed to drink tap water ad libitum. All the treatments and procedures for the rats were approved by the animal care and use committee of the Meir Hospital.

The rats were divided into four groups: (1) normal pregnant rats (P rats; \( n = 13 \)); (2) virgin rats treated chronically with insulin (V-INS rats; \( n = 8 \)); (3) pregnant rats treated chronically with insulin (P-INS rats; \( n = 20 \)); (4) P-INS rats treated with L-arginine from day 11 of gestation (L-ARG rats; \( n = 15 \)).

Insulin-treated rats received a 2 mm segment of a 7 mm sustained-release insulin implant (linplant; Linshin Canada Inc., Scarborough, Ontario, Canada) designed to deliver approx. 2 units/day for more than 40 days by means of a 12-gauge hypodermic needle under brief ether anaesthesia. A pellet was implanted subcutaneously 1 week before and on day 7 of pregnancy. Administration of two pellets, with a moderate amount of insulin in each one, allows the gradual development of hyperinsulinemia without severe hypoglycaemia. Control rats underwent sham implantations under the same conditions [2]. L-Arginine was given orally (in drinking water) at a dose of 2 g/l, a dose that reverses the hypertensive effects of \( \text{NO}^- \)-nitro-L-arginine methyl ester (NO synthase inhibitor) in pregnant rats, and normalizes mean arterial pressure in hypertensive pregnant rats with adriamycin nephropathy [3]. The arginine content in the food was 11.7 mg/g. In the pregnant groups, food intake increased from 20 ± 0.5 g/day before pregnancy to 26 ± 0.7 g/day at day 20 of pregnancy (no difference between groups).

Accordingly, the daily intake of arginine in the food increased from 234 to 304 mg. In the L-ARG group, the daily intake of water increased throughout pregnancy from 22 ± 1.4 to 31 ± 1.5 ml. Therefore the extra arginine intake in this group increased from 44 to 62 mg/day, i.e. 20% more than in P-INS and P rats.

Day 1 of gestation was documented by the presence of spermatozoa in the vaginal smear. Measurement of systolic blood pressure (SBP) and 24 h urine collections were performed before mating and on days 19–20 of pregnancy (duration of pregnancy ~ 22 days); the urine collections were used for measurement of urinary noradrenaline (norepinephrine), microalbuminuria and urinary metabolites of nitrate (UNO₃). Plasma glucose, creatinine, fructosamine, endothelin-1 (ET-1) and insulin were measured before and at the end of pregnancy. On day 22, the usual day of delivery, the rats were killed, and the number and weight of the fetuses were determined. Five P-INS rats were not killed, in order to measure SBP 30 and 60 days after delivery.

The 24 h urine collections were obtained using individual metabolic cages. On each occasion, the rats were put in metabolic cages for 2 days and received a diet low in nitrate (7 \( \mu \text{mol} / 100 \text{g} \)). Gentamycin (6 mg/tube) was added to each test tube of urine, to prevent bacterial contamination. SBP was measured in awake rats, by tail cuff manometry using a Narco Bio system automated sphygmomanometer. To ensure that the rats rested quietly during the blood pressure measurements in the Plexiglas constraining cages, they were systematically trained prior to each measurement. SBP was measured 10 times in each rat. Of the 10 recordings, the first three were discarded and the mean of the last seven was the value taken.

Non-fasting serum creatinine, glucose and fructosamine levels were measured by standard methods. Microalbumin concentrations in the urine were measured by immunoturbidimetry in a Kongi-Progress automatic analyser by using monoclonal anti-(rat albumin) antibody (Binding Site, Birmingham, U.K.). Rat albumin standard was prepared from rat serum calibrator. Urinary noradrenaline was extracted from urine on acid-washed alumina and determined by HPLC on a Bondapack reverse-phase column (Beckman, Fullerton, CA, U.S.A.) with electrochemical detection [4]. Plasma ET-1 levels were determined by RIA kit (Nichols Institute Diagnostics Ltd, Wijchen, The Netherlands) after activation on Sep-Pak C18 cartridges (Waters, Milford, MA, U.S.A.). Urinary nitrate (NO₃) was determined by an enzymic end-point method, using nitrate reductase from Aspergillus sp. (Sigma), and is expressed as NO₃/\( \mu \text{g} \) of creatinine. The decrease in absorbance at 340 nm as a result of the oxidation of \( \beta \)-NADPH was recorded. FAD was used as a supplementary electron carrier [5]. All the measurements were registered using an automatic spectrophotometer. Results from 24 h urine collections are expressed as \( \mu \text{mol} \cdot \text{day}^{-1} \cdot \text{mg}^{-1} \) creatinine.

Results are expressed as means ± S.E.M. Differences between groups were assessed by ANOVA. Non-parametric tests or Student’s \( t \)-test for paired groups were used as appropriate. Statistical analysis was carried out on an IBM PC using the Crunch statistical package, 4th edition (Crunch Software Co., Oakland, CA, U.S.A.); differences were considered significant at the \( P < 0.05 \) level.

RESULTS

Clinical findings

The increase in body weight was similar in all pregnant rats, with a slight tendency to be lower in P-INS rats
Table 1  Clinical and biochemical metabolic parameters at the beginning and end of pregnancy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before/ end of pregnancy</th>
<th>P rats</th>
<th>L-ARG rats</th>
<th>V-INS rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>Before</td>
<td>244 ± 5</td>
<td>250 ± 4</td>
<td>247 ± 4</td>
</tr>
<tr>
<td></td>
<td>End</td>
<td>316 ± 9</td>
<td>316 ± 7</td>
<td>325 ± 6</td>
</tr>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>Before</td>
<td>106 ± 7.4*</td>
<td>83 ± 6</td>
<td>84 ± 5.8</td>
</tr>
<tr>
<td></td>
<td>End</td>
<td>116 ± 6.8*</td>
<td>74 ± 4</td>
<td>66 ± 2.8</td>
</tr>
<tr>
<td>Serum fructosamine (mg/dl)</td>
<td>Before</td>
<td>113 ± 1.7</td>
<td>118 ± 0.8</td>
<td>117 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>End</td>
<td>130 ± 3.2*</td>
<td>109 ± 3.5</td>
<td>104 ± 2.1</td>
</tr>
<tr>
<td>Serum albumin (g/dl)</td>
<td>Before</td>
<td>3.5 ± 0.08*</td>
<td>2.66 ± 0.06</td>
<td>2.52 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>End</td>
<td>50 ± 5</td>
<td>116 ± 7</td>
<td>166 ± 10</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>Before</td>
<td>1.05 ± 0.07</td>
<td>1.01 ± 0.05</td>
<td>1.04 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>End</td>
<td>1.54 ± 0.05†</td>
<td>1.19 ± 0.08</td>
<td>1.23 ± 0.05</td>
</tr>
<tr>
<td>24 h UNO excretion (μmol·l⁻¹·mg⁻¹ creatinine)</td>
<td>Before</td>
<td>12.7 ± 1.1</td>
<td>14.8 ± 1.4</td>
<td>13.2 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>End</td>
<td>42.4 ± 3.3†</td>
<td>22.1 ± 2.3</td>
<td>28.1 ± 1.9†</td>
</tr>
<tr>
<td>Urinary noradrenaline (ng/mg of creatinine)</td>
<td>Before</td>
<td>218 ± 33</td>
<td>241 ± 22</td>
<td>178 ± 17</td>
</tr>
<tr>
<td></td>
<td>End</td>
<td>322 ± 28</td>
<td>332 ± 41</td>
<td>301 ± 23</td>
</tr>
<tr>
<td>Plasma ET-1 (pg/ml)</td>
<td>End</td>
<td>1.67 ± 0.2</td>
<td>2.1 ± 0.4</td>
<td>2.4 ± 0.5</td>
</tr>
</tbody>
</table>

Figure 1  Changes in SBP during pregnancy

- □, SBP before pregnancy; ■, SBP at the end of pregnancy. Values are means ± S.E.M. Significance of differences: *P < 0.05 compared with P rats before pregnancy; **P < 0.01 compared with the same group before pregnancy and with P rats at the end of pregnancy; ***P < 0.01 compared with the same group before pregnancy and with P-INS rats at the end of pregnancy.

The initial and final values for the urinary excretion of noradrenaline were equivalent in all groups of pregnant rats. As reported previously, the urinary excretion of

**SBP values**

Pre-gestational values of SBP were equivalent in all groups. At the end of pregnancy in P-INS rats, SBP had increased from 97 ± 1.5 to 104 ± 3 mmHg (P < 0.05). In P rats, SBP decreased from 97 ± 1.8 to 82 ± 3.5 mmHg (P < 0.05 compared with values before pregnancy, and P < 0.01 compared with P-INS rats at the end of pregnancy) (Figure 1). In the L-ARG group, SBP decreased from 97 ± 1.7 to 83 ± 2.3 mmHg (P < 0.01) (Figure 1). The percentage changes in SBP of L-ARG rats were similar to those of the P animals, and significantly different from those of the P-INS group (−14% for L-ARG, −15% for P and +10% for P-INS rats; P < 0.01). In the five P-INS rats that were not killed, SBP values measured 30 and 60 days after delivery had returned to pre-gestational values (92 ± 4 and 94 ± 4.6 mmHg respectively). A direct positive correlation was found between SBP and plasma insulin levels (r = 0.52, P = 0.02, df = 20).

**Laboratory results (Table 1)**

Plasma insulin levels were higher in P-INS, L-ARG and V-INS rats compared with P rats. No severe hypoglycaemia developed during the study, as shown by both serum glucose and fructosamine measurements. At the end of pregnancy creatinine clearance was augmented in P rats, but remained unchanged in P-INS and L-ARG rats. A significant negative correlation was found between creatinine clearance and plasma insulin levels (r = −0.51, P = 0.002, df = 18).

The initial and final values for the urinary excretion of noradrenaline were equivalent in all groups of pregnant rats.
In the present study, rats with chronic hyperinsulinaemia had higher blood pressure values at the end of gestation. The pathogenic mechanisms of such changes are unknown. Increased activation of the sympathetic nervous system has been proposed, but this remains controversial [9,10]. The urinary excretion of noradrenaline in the present study was not different in P-INS rats compared with P animals. These data do not support the presence of increased activity of the sympathetic nervous system; however, given that extremely variable changes in plasma concentrations and urinary excretion of catecholamines have been reported in hypertensive pregnancies [11–13], no definitive conclusion can be reached.

ET-1, a well known potent vasoconstrictor, may be involved in the pathogenesis of hypertension, as seen in obese patients with syndrome X [7,8]. In the present study, rats with chronic hyperinsulinaemia had higher blood pressure values at the end of gestation. The pathogenic mechanisms of such changes are unknown. Increased activation of the sympathetic nervous system has been proposed, but this remains controversial [9,10]. The urinary excretion of noradrenaline in the present study was not different in P-INS rats compared with P animals. These data do not support the presence of increased activity of the sympathetic nervous system; however, given that extremely variable changes in plasma concentrations and urinary excretion of catecholamines have been reported in hypertensive pregnancies [11–13], no definitive conclusion can be reached.

ET-1, a well known potent vasoconstrictor, may be involved in the pathogenesis of hypertension in pregnancy [14,15]. The relationship between insulin and ET-1 is not clear. In in vitro experiments, insulin stimulated the synthesis of ET-1 by endothelial cells [16,17], but in in vivo studies insulin did not affect blood levels of ET-1 [18]. In the present work the plasma levels of ET-1 in P-INS rats were similar to those in P and l-ARG rats. Therefore, as a circulating hormone, ET-1 does not seem to play a major role in the development of hypertension in P-INS rats. However, we cannot exclude the possibility that insulin may stimulate the synthesis of ET-1 by the placental tissue, and thereby interfere with fetal growth [14].

The urinary excretion of nitrate increased during normal pregnancy, and this was partially blunted in the P-INS rats, confirming our previous result [2]. The measurement of urinary nitrate or UNO₃ is a useful index of NO synthesis that must, however, be interpreted carefully. Nitrate intake was particularly well controlled in our study, suggesting that the observed changes in UNO₃ excretion may be of significance. Even under these conditions, however, it has been suggested that UNO₃ does not reflect systemic biologically active NO adequately [19]. In order to evaluate the implications of changes in NO production in the pathogenesis of hypertension in P-INS rats, an NO precursor (l-arginine) was added to the diet from mid-pregnancy. In hyperinsulinaemic pregnant rats, treatment with l-arginine resulted in an increase in UNO₃ excretion and a significant fall in SBP, with values becoming similar to those found in P rats. Therefore a blunted increase in NO synthesis in P-INS rats may have played a pathogenic role in the observed increase in blood pressure. The reason why NO synthesis may have been blunted is unknown. Activation of the sympathetic nervous system (as discussed above) or of the renin/angiotensin system has been shown to be involved in the pathogenesis of hyperinsulinaemia-induced hypertension [20].

In addition to the elevated blood pressure, the gestation of hyperinsulinaemic rats was complicated by intrauterine growth retardation. Maternal hyperinsulinaemia/insulin resistance can be associated with fetal growth retardation [21]. In the present study, treatment with l-arginine improved fetal weight without any changes in the levels of plasma insulin or serum glucose. These data suggest a direct effect of NO on fetal weight, due either to an amelioration of the feto-placental circulation or to unknown l-arginine-related mechanisms.

REFERENCES


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Received 29 September 2000; 5 February 2001; accepted 22 March 2001